SPECTRAL SENSITIVITY OF THE RETINAL ACTION POTENTIAL OF COLIAS

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I. INTRODUCTION

Color vision depends on the ability of the visual system to appreciate the distinction between at least two different wavelengths of light independently of their intensity. When studying the capacity of an organism's visual system to make this distinction, one must be able to control the physical properties of the stimulus at the cornea while monitoring a response at some point along the afferent pathway from first order neuron to the higher visual centers. Behavioral responses to differential wavelength stimulation may be studied if one is interested primarily in the significance of color to the organism. As a technique for monitoring the properties of the receptor system, however, behavioral responses are inadequate, for information may be lost or reorganized in transfer to the central nervous system or in execution by the organism. This study concerns itself with the ability of the primary visual cells to transduce physical energy into generator potentials, and thus to determine at the earliest point in the receptor pathway the capacity of the organism for wavelength discrimination.

In general it has been found that the spectral sensitivity range of insects is shifted 100nm towards shorter wavelengths in relation to man's range. Thus while man is maximally sensitive to blue, green and red, the insect, it appears, appreciates best ultraviolet, blue and green. Some evidence, however, most of it equivocal, indicates that certain insects may have a sensitivity peak in the red

region. This study offers some evidence concerning the differential sensitivity of the first order neurons of the butterfly Colias to red light, and in addition measures the spectral sensitivity in the ultraviolet, blue and green regions. If an additional sensitivity peak in the long wavelength end of the visible spectrum could indeed be proven, this would be the first organism reported with a "tetrachromatic" receptor system.

A. FUNDAMENTAL CONCEPTS

1. Intensity and Wavelength

The initiating step in the visual process is the absorption of light by the photolabile visual pigment within the receptor cell. At some point in the degradation of this pigment the receptor potential is formed. The subsequent frequency of firing of the axon of the second order neuron is related to the magnitude of the receptor potential. Consequently the response of the cell is dependent not only on the absorption coefficient of the pigment but also on the energy content of the stimulus at each wavelength. Thus given a receptor field homogeneous in respect to the absorption spectrum of the visual pigment contained in the receptor cells, whether this absorption spectrum has one or several maxima, the field will be unable to differentiate between a low intensity light at a wavelength of maximal absorption and a strong light at a wavelength of lesser absorption. Wavelength and intensity are thus not independent parameters and such a field will be color blind. If on the other

hand a receptor field is heterogeneous in respect to the absorption spectra of the visual pigments contained in different cells, it may be able to respond differentially to monochromatic stimulation independently of intensity. A spectral sensitivity curve reveals the limits of the visible spectrum, but unless its maxima can be demonstrated to arise in separate cells it does not prove the existence of wavelength discrimination.

2. The Action Spectrum and the Equal Energy Spectrum

The differential sensitivity of a single receptor cell or group of cells to a range of wavelengths is expressed as a spectral sensitivity curve (action spectrum). The log of the reciprocal of the relative number of photons necessary to elicit equal-sized criterion responses is plotted at each wavelength.

Another commonly used expression of the differential wavelength sensitivity of the eye is the "spectral efficiency curve". This curve describes the size of the response elicited at each wavelength when the eye is stimulated with an "equal energy spectrum" (flashes of light of equal energy at each wavelength). The maxima of this curve are broader than those of the corresponding action spectrum because "physiological responses are nonlinear functions of stimulus energy" (Goldsmith, 1964).

The spectral efficiency curve does not approach the ideal of reflecting the absorption spectrum of the receptor molecule because it does not describe the amount of energy necessary at each wavelength to achieve a given amount of photochemical reaction, as measured by

the receptor potential. The action spectrum, in describing energy requirements for constant excitation, approaches the ideal of expressing the absorption spectrum of the receptor molecule. The assumption must still be made, however, that there is a constant correlation between the photochemical reaction and initiation of the receptor potential, i.e., that the quantum efficiency of excitation does not vary with wavelength.

B. CONTRIBUTIONS OF BEHAVIORAL TECHNIQUES

Several different methods, both behavioral and electrophysiological, have been used to study the color vision of insects. It is worthwhile reviewing first some of the behavioral methods as a means of illustrating the principles which are integral to an understanding of color vision in general. Although the spontaneous preference of insects for certain colored flowers had long been noted, the argument was still strong in 1913 (von Hess) that such discrimination was based on relative brightness differences of the preferred colors. The controversy was settled that same year by von Frisch. He trained bees to search for food on blue paper. When the food was removed, the bees returned to the blue paper, now placed amidst gray papers of all reflectances, thus conclusively demonstrating their ability to distinguish blue from a spectral mix of the same brightness.

Hamilton (1922) was the first to utilize the phototactic response in studying color vision. He adjusted the intensities of two different

colored lights until equal numbers of Drosophila were attracted to each light. He observed that if the group was first exposed to one of the two lights, the majority of individuals would then be attracted to the other color when allowed to choose between the two. This experiment illustrates the technique of selective adaptation, first used by von Frisch and Kupelwieser (1913) in investigating crustacea. It has been a powerful tool in visual physiology because of its action in increasing the relative prominence of the response from the spared receptors.

Schlieper (1927) used optomotor responses as a technique for testing the capacity of untrained animals to distinguish between different wavelengths independently of their intensity. He observed the responses of crustacea to a rotating drum banded with alternate stripes of colored and gray papers. For each color he was able to find a gray of a reflectance such that no optomotor response was elicited. Von Buddenbrock and Friedrich (1933) improved upon this method, using crabs. They found that two colors, each of which when alternated against reference grays of equal brightness gave no response, did indeed elicit an optomotor response when alternated against one another. Proof of the existence of color vision was thus extended to crustacea by satisfying the maxim of wavelength discrimination independent of brightness. Schlieper's experiment illustrates that while the existence of a criterion behavioral response may be sufficient proof of color vision, its absence only demonstrates the inability of the test wavelength to elicit the specific response.

Information may be lost in transfer from visual cell to somatic musculature. The experiment of von Buddenbrock and Friedrich invokes the principle of colorimetry in a somewhat rudimentary form. Colorimetry states that for each test color a reference wavelength or group of wavelengths can be found which when combined in appropriate proportions will be indistinguishable from the test wavelength. One or more of the reference wavelengths in some instances may have to be added to the test light to achieve a match. In theory this principle can be used to determine the minimum number of color receptors in an eye.

Daumer (1956) refined the use of colorimetry by illuminating feeding dishes from below with pure spectral lights. Worker bees were trained to collect sugar syrup from a dish colored by light of known intensity and wavelength. They were then presented a second dish illuminated by a wavelength or combination of wavelengths equal in energy to the light under the training dish. The wavelength combinations were adjusted until equal numbers of bees were attracted to the two dishes, i.e., until a colorimetric match was obtained. He found that the white light of a xenon arc (emission spectrum approximately 300 - 700 nm) could be matched by an appropriate combination of the three reference wavelengths of blue (440 nm), ultraviolet (360 nm) and yellow (588 nm). In addition, for each wavelength in the visible spectrum of the bee a colorimetric match could be obtained by appropriate mixtures of one, two or three of these reference wavelengths. This data stands as the best

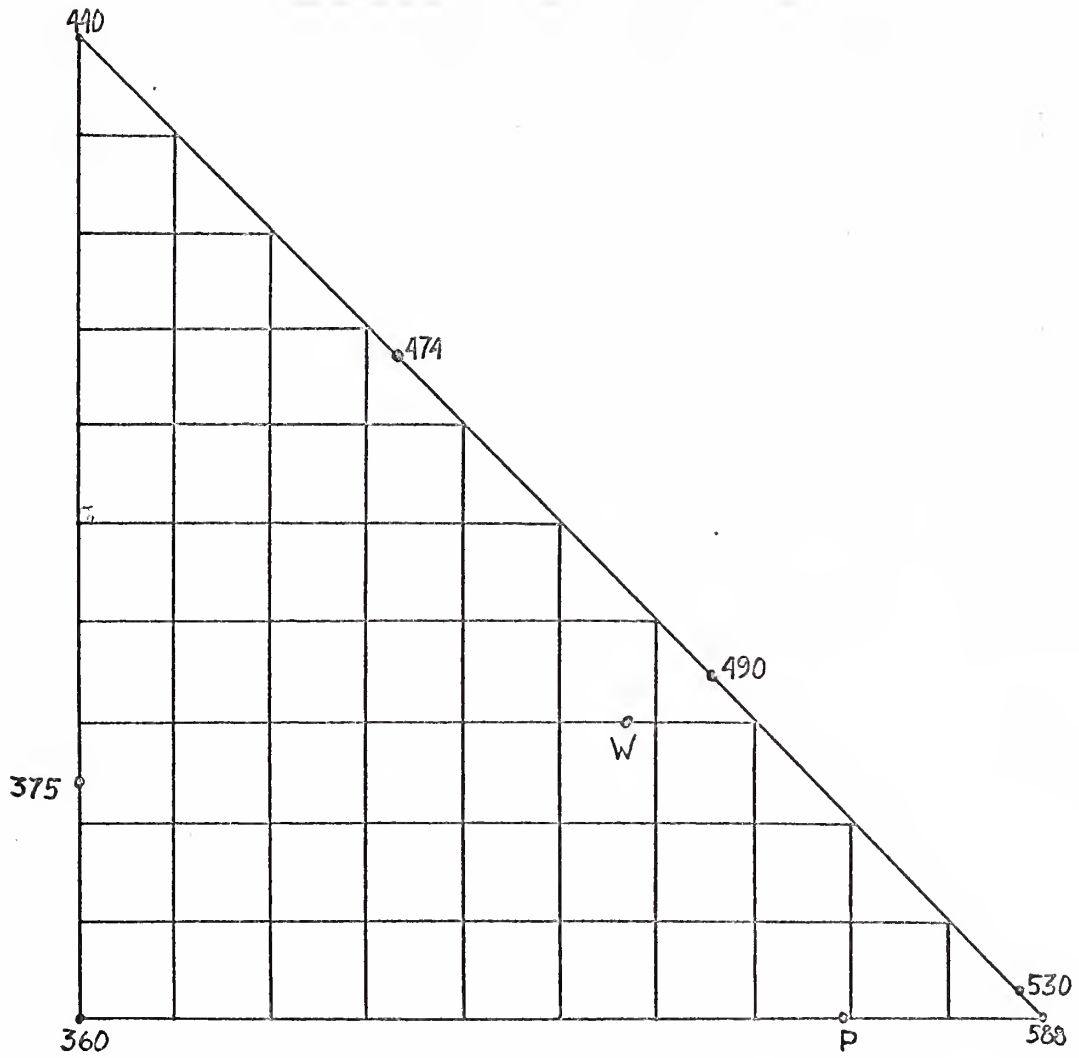

FIGURE 1. Tentative chromaticity diagram of the worker honeybee. "W" represents the white point, the equivalent of the emission spectrum of the sun or a xenon arc; "P" represents "bee's purple", complementary to 440 nm. Drawing modified from Goldsmith (1961a); based on data given by Daumer (1956).

behavioral evidence for a visual system in the bee mediated by three receptors, each maximally sensitive at a different wavelength. From the experiments of Daumer, Goldsmith (1961a) was able to construct a chromaticity diagram for the bee (Figure 1). The three primary colors are placed at the corners of the triangle.

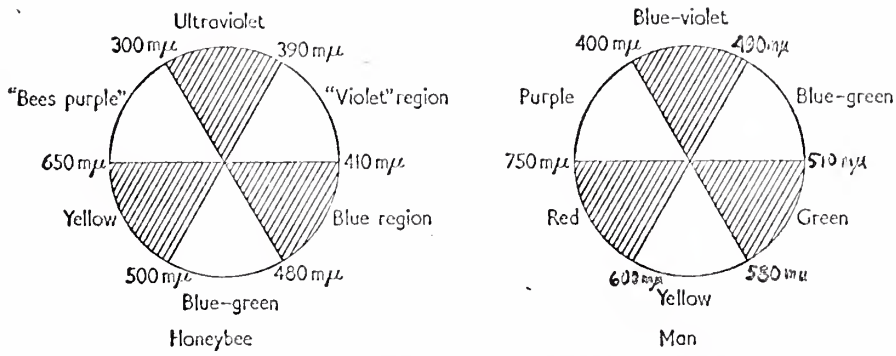

FIGURE 2. Color circle of man and worker bee. Shaded areas represent the primary colors. (From Daumer, 1956)

The test wavelengths colorimetrically matched by two of these colors are placed along the line of the periphery connecting them. Thus, for example, a 474 nm light can be matched by mixing 23% yellow (588 nm) with 67% blue (440 nm). A light requiring a trichromatic mix, for example white, is plotted at the appropriate position in the middle of the triangle. This data strongly supports a trichromatic theory of color vision in the bee. For each of the primary colors Daumer determined the spectral complement. From this data he constructed a color circle for the bee (Figure 2) analogous to the color circle for man. By mixing ultraviolet and yellow he found a color complementary to blue. This color, not distinctly appreciable to man, he named "bee's purple" because like "human purple" it represents a mix of the primary colors at either end of the visible spectrum. Study of this diagram supports the concept that the insects' visible spectrum is shifted approximately 100 nm to shorter wavelengths as compared to man. Although

Daumer's data indicate that the bees' spectral range extends up to 650 nm, no evidence is offered for the presence of a receptor with a sensitivity maximum in this region.

C. CHARACTERISTICS OF THE ELECTROPHYSIOLOGICAL RESPONSE

By basing one's analysis of spectral sensitivity on potential changes within the retina, the mediating influence of central integrative mechanisms on the measured response may be reduced or eliminated. One may thus obtain quantitative data on the relative size and position of the sensitivity maxima of the receptors.

Of particular interest is the relative energy requirements of different wavelengths in eliciting equal sized potential changes across the limiting membrane of individual receptor cells. An analysis of the action spectra of differing receptor types will yield a clear picture of the spectral sensitivity of the retina as a whole.

Alternatively, one may obtain data pertinent to the spectral sensitivity of individual receptors by analyzing the differential wavelength dependence of the mass response (ERG). The ERG is the record of potential changes between a subcorneal electrode and a reference electrode proximal to the basement membrane, and as such represents a composite of the responses of the individual retinular cells falling in the path of the stimulating light. The magnitude of the mass response depends not only on the size of the response of each individual receptor cell stimulated, but, in

addition, on the number of cells stimulated.

A screening pigment which has an unequal absorption spectrum over the range of wavelengths tested will affect the number of cells stimulated at certain wavelengths and thus the shape of the action spectrum. Two populations of receptors must be recognized: one whose ommatidial axes are parallel to the beam of the stimulating light, and another whose axes lie at an oblique angle. The magnitude of the response of the former group is independent of any effects of the pigment screen. The latter group, whose axes are oblique to the test light, will contribute to the mass response at wavelengths at which the pigment screen is transparent. The size of the response at such wavelengths will thus be disproportionately large, and a false sensitivity maximum will appear on the spectral sensitivity curve. By measuring the absorption spectrum of the screening pigment one may assess the significance of the elicited sensitivity maxima. Additionally, in flies, the size of the off-effect (a response of second order neurons) is a function of the number of receptors responding, and therefore gives a clue to the presence of a screening effect (Goldsmith, 1965). In bees, however, where there is no screening effect, the off-effect also increases at long wavelengths. The efficacy of the off-effect as a measure of recruitment must therefore be determined independently in each animal. A third technique useful in determining the presence of a screening effect is comparison of the waveforms elicited when

(a) only several facets are illuminated, by means of a microspot of light, and (b) the entire cornea is illuminated. If the ratio of sizes of off-effect/receptor component is larger in (b) than (a), a screening effect is very likely present (Goldsmith, 1965).

Since the rules of combination which govern the way in which the receptor potentials of individual cells combine to produce the mass response are not known, it is difficult to extrapolate backwards from the action spectrum of the eye as a whole to determine the sensitivity curves of individual cells. The location and relative size of sensitivity peaks, however, may be evident from analysis of the action spectrum of the eye. A powerful tool in accentuating these maxima and selecting out the contributions of individual receptors is the technique of selective adaptation, first used in 1913 by von Frisch and Kupelwieser in behavioral experiments. The eye is adapted with a color spanning one of the maxima of the action spectrum. The photopigment with absorption in the range of the adapting light will be bleached, and the threshold of the cell containing this pigment will rise. The responses of the spared receptors will become relatively more prominent and the corresponding maxima in the action spectrum will be accentuated. Depression of two peaks by selective adaptation of one indicates the presence of a photopigment with double absorption maxima. The crucial variable in differential wavelength sensitivity is the number of photoreceptors capable of responding independently of one another. Thus a single

cell which contains two photopigments with different absorption spectra, or one photopigment with a double peaked absorption spectrum, is incapable of responding differentially to the two wavelengths of maximal absorption. Selective adaptation can thus be used to identify the number of different types of photoreceptors, and to indicate the situation in which two maxima in an action spectrum arise from a single photoreceptor. Maxima which are the result of a screening effect, however, are treated as if they arose from distinct receptor types; separate techniques must be employed, therefore, to determine whether the peaks which are singled out by selective adaptation indicate the presence of distinct receptor types or are secondary to a screening effect.

By careful adherence to the considerations just discussed, it is possible by means of the mass response to construct a spectral sensitivity curve which accurately reflects the location and relative prominence of the various sensitivity maxima.

D. SPECTRAL SENSITIVITY: OUR CURRENT STATE OF KNOWLEDGE

1. The Honey Bee

The honeybee represents a striking example of an insect in which knowledge concerning the location and relative heights of the sensitivity maxima, as well as the number of photosensitive pigments, was first worked out from analysis of the retinal action potential, and later confirmed by intracellular recording.

The action spectrum of the drone over the spectral range

400-650 nm exhibits a sensitivity maximum at 440 nm (Goldsmith, 1958b). This peak corresponds to the absorption maximum of the photopigment extracted from the heads of workers (Goldsmith, 1958b). In addition, the drone eye contains maxima at 340 nm and 540 nm (Goldsmith, 1961b). A prominent feature of the action spectra of drone eyes is that the relative heights of the elicited sensitivity maxima vary markedly from one preparation to the next; this is a function of which particular ommatidial units happen to be oriented parallel to the axis of the test beam, and indicates that the relative proportions of receptor types varies throughout the eye. When a test light with a spectral range extending into the ultraviolet was employed in the measurement of an action spectrum of the worker eye, sensitivity maxima were found at 340 nm and 535-540 nm (Goldsmith, 1960). The green peak was relatively more prominent than the ultraviolet. Attempts by the same author to estimate the spectral sensitivity curve of this green receptor by selectively adapting the eye with an ultraviolet light met with limited success. The log relative sensitivities of both the 340 nm and 540 nm peaks were decreased by ultraviolet adaptation, indicating that the photopigment mediating green sensitivity has significant absorption in the ultraviolet range. Adaptation of the eye with green light, on the other hand, depressed the long wavelength receptor and produced a sharp maximum of sensitivity at 340 nm, representing the spectral sensitivity curve of the ultraviolet receptor. The interpretation of this data is that two receptors are present in the worker eye. One of these is maximally sensitive in the ultraviolet, and the other in the green with secondary

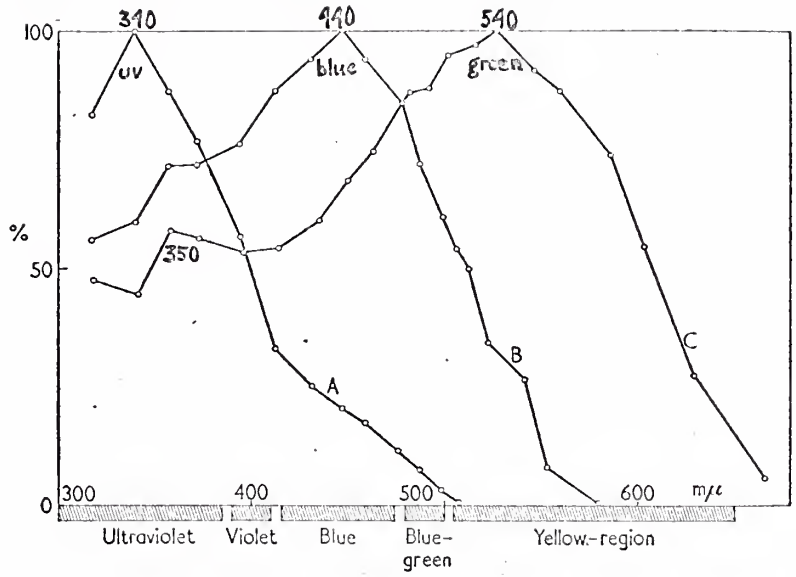

FIGURE 3. Equal energy spectra of three individual cell types in the compound eye of the drone bee. Ordinate equals percent of largest response for each receptor type. Along the abscissa are shown the spectral ranges of five of the six colors distinctly visible to the worker bee (Daumer, 1956) as shown in Figure 2; note that in regions of rapidly changing hues, e.g., the violet and blue-green, the curves are crossing one another. (From Autrum and von Zwehl, 1963)

ultraviolet absorption.

The validity of this data was confirmed by determination of spectral efficiency curves for single visual cells in the eyes of worker and drone bees (Autrum and von Zwehl, 1962; Autrum, 1963). The most frequently punctured cell in the worker exhibited maximal sensitivity at 540 nm; its sensitivity dropped off sharply at longer wavelengths, but rose to a secondary maximum in some cases at 350 nm (Figure 3), predicted by Goldsmith's extracellular work. Less frequently encountered was another type of receptor cell with maximal sensitivity at 340 nm, dropping off sharply at both longer and shorter wavelengths. In addition, a third cell type with a

maximum at 440 nm was found by these investigators; although found in the drone by Goldsmith (1960) its presence in the worker eye had been hitherto undetected by neurophysiological methods. In the drone they found a similar pattern of sensitivity maxima, although here (Figure 3) the blue receptor (as well as the green) exhibited a secondary sensitivity maximum in the ultraviolet region. Their curves have a broader peak at 440 nm than either the absorption maximum of the photopigment or the spectral sensitivity function at this wavelength. This is because an equal energy spectrum is based on the size of the response, which is a nonlinear function of stimulus energy, as discussed earlier. Autrum's experiments present the first evidence at the single-cellular level of a trichromatic system of wavelength discrimination; the trichromatic theory of color vision for the bee was first predicted on behavioral grounds by Daumer in 1956.

2. The Fly

In contrast with the experience in the bee, the action spectrum elucidated by analysis of the extracellular response could not be confirmed by single cell work. Only later when Goldsmith (1965) conclusively demonstrated the errors of analysis of the mass response which had led to the postulation of a red receptor was the issue settled.

The ERG of the Dipteran eye consists of an initial positive transient (the "on-effect"), a maintained negative component

(the "receptor component") and a final negative transient (the "off-effect"). In order to interpret action spectra based on these components it is important to understand their origins. If the retinular cell bodies are electrically isolated by placement of an active electrode among the retinulae and a reference electrode at the cornea, a response devoid of transients is obtained (Ruck, 1961), similar in shape to the maintained component of the ERG. Further evidence that this component may properly be termed a "receptor component" is provided by experiments in which the retinular cells are isolated from second order neurons by surgical removal of the optic ganglion (Bernhard, 1942; Jahn and Wulff, 1942; Autrum, Autrum, and Hoffmann, 1961; Eichenbaum, 1967). The response obtained is again devoid of transients, thus supporting Ruck's suggestion that the maintained component of the ERG is in fact a summation of receptor potentials arising across retinular cell membranes. Intracellular records from retinular cells confirm this interpretation and show furthermore that the off-effect is postsynaptic in origin (Burkhardt and Autrum, 1960; Naka, 1961; Naka and Eguchi, 1962a,b).

The physiological techniques which have been used in studying differential wavelength sensitivity in Dipteran eyes may generally be divided into three groups: mass response to test flash, single cell response to test flash, and mass response to heterochromatic flicker. Although the true spectral sensitivity

of the Dipteran eye became evident with the development of intracellular recording methods, some effort will be expended here in elucidating the mistakes in analysis by early workers of the mass response to test flash and heterochromatic flicker. This analysis will lead to an understanding of the effects of the screening pigment and the meaning of the off-effect, and will provide some guide rules to be used in interpretation of the results of the present experiments on the butterfly.

(a.) mass response to test flash:

Autrum (1955) found sensitivity peaks in the spectral efficiency curve of the on-effect of Calliphora erythrocephala at 340 nm, 490 nm and 630 nm. With increasing intensities the green peak shifted towards 520 nm and the red peak grew progressively more prominent. The white-apricot mutant, which is devoid of the red ommochrome screening pigment, lacked both the red peak and the green shift. Three to four log units less energy were necessary in the white eye as compared with the red eye to produce equal sized green peaks. Based on these experiments, Autrum was the first to suggest that the red peak was an artifact produced in wild-type flies by the decreased absorption of the ommochrome screening pigment at long wavelengths. The absorption spectrum of ommochrome pigment granules from wild-type Musca, measured with a microspectrophotometer (Strother, 1966), has in fact been demonstrated to exhibit a sharp drop-off around 600 nm.

Walther and Dodt (1957, 1959) found sensitivity peaks for the receptor component in Calliphora erythrocephala at 340 nm, 507 nm and 630 nm, although they noted increasing prominence of the red peak with increasing intensity. Autrum's experiments on the white-apricot mutant were repeated by Hoffmann and Langer (1961), together with experiments on the chalky mutant, which lacks the yellowish pteridine as well as the ommochrome screening pigment. Both had peaks at 360 nm and lacked the red peak; the white-apricot exhibited a green peak at 500 nm in agreement with Autrum, while the green peak for the chalky mutant was shifted to 510 nm. Autrum, Autrum and Hoffmann (1961), in further experiments on Calliphora erythrocephala noted a wavelength dependence of the mass response; the on- and off-effects became increasingly prominent as the wavelength of the test flash was advanced from green to red.

In summary, comparison of the action spectra of red and white eyes suggests that the red peak is an artifact produced by selective transmission of long wavelengths by the screening pigment. However, on the assumption that the wavelength dependence of the off-effect indicates responses of different kinds of cells, one could argue that the increased prominence of the off-effect at long wavelengths supports the red receptor hypothesis. As will be shown later, this assumption is incorrect, for in flies the size of the off-effect is a function of the number, rather than the kind of receptors stimulated.

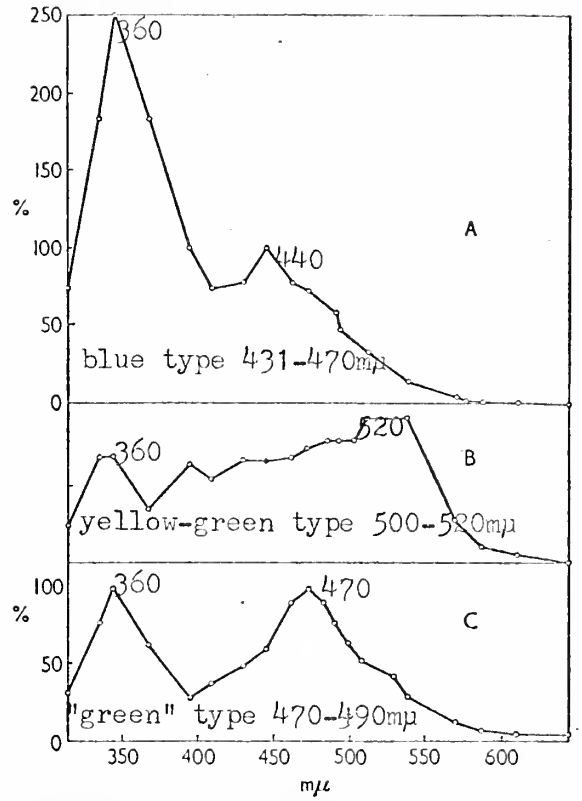

FIGURE 4. Equal energy spectra of three individual cell types in the compound eye of Calliphora. Ordinate equals percent of largest response in visible spectrum. (A) blue type: note prominent uv peak. (B) yellow-green type: uv peak lower than visible peak. (C) "green" type: uv and green same height with minimum at 400 nm; accounts for five-sevenths of cell population in ventral part of eye; only cell type dorsally. (Modified from Burkhardt, 1962).

(b.) single cell response to test flash:

The equal energy spectra of single cells in the blowfly Calliphora erythrocephala indicate (Figure 4) the presence of three different kinds of receptors (Autrum and Burkhardt, 1960, 1961; Burkhardt, 1962). Every cell has a peak at 345 nm and in addition a second peak in the range 431-542 nm. The middle-wavelength peaks cluster at 431-470 nm, at 470-490 nm and at 500-520 nm;

accordingly these cell types are called blue, "green" and yellow-green. The "green" cell type is the most common, and the only cell in the dorsal half of the eye; the ultraviolet and "green" peaks are equal in height, and there is a sharp minimum between them at 400 nm. The blue cell type has an ultraviolet peak significantly higher than the blue peak; the yellow-green cell type has an ultraviolet peak slightly lower than the 520 nm peak, and the sensitivity at intermediate wavelengths is not impressively lower than either peak.

All three cell types have sensitivity at wavelengths greater than 600 nm, although in no case was a peak at a wavelength greater than 542 nm elicited from receptors directed towards the light source. Some sensitivity curves of directed receptors fit closely the curve obtained from the chalky mutant based on the receptor component of the mass response, elicited by Hoffmann and Langer (1961). The presence of a peak at 616 nm in a few individual cells which gave small responses, and were not directed towards the light source, lends further support to the hypothesis that the red peak in the mass response is secondary to a screening effect. This observation provides strong evidence against the presence of a red receptor in this insect, and indicates that the screening hypothesis is indeed correct, as originally suggested by Autrum in 1955.

Selective adaptation of either the blue or the "green" cell types with 345 or 490 nm light produces no change in the shape of the equal energy spectra (Burkhardt and Hoffmann, 1962). This

suggests that the ultraviolet, and blue or "green", sensitivity in these receptors is mediated by a single photosensitive pigment. The question of whether this eye possesses a distinct ultraviolet receptor is dependent on one's interpretation of the sensitivity curves; however the great relative prominence of the ultraviolet peak in the 490 nm receptor suggests that functionally this insect is equipped to distinguish ultraviolet and short wavelengths (as opposed to wavelengths greater than 500 nm) with ease. This is particularly true in the dorsal half of the eye where only the 490 nm receptor is present; the animal is therefore color blind to light striking the upper part of its eye, yet is exquisitely sensitive to ultraviolet over this area. Similar color blindness was noted dorsally in Notonecta (Rokohl, 1942; Lüdtke, 1954), ventrally in Periplaneta (Walther, 1958a, b), and in varying regions of the eye of Libellula (Mazokhin-Porshnyakov, 1959); these findings point up the necessity for noting in which part of the eye one places the electrode when recording mass responses.

Burkhardt (1962) noted that of 108 cells tested approximately five-sevenths of these were the "green" receptor type, and the remainder were equally divided between the blue and yellow-green types. Since he thought that each ommatidium in Diptera eyes is composed of seven retinular cells (Dietrich, 1909; Sato, 1950; Fernández-Morán, 1958), he speculated that five of these cells contained the "green" photosensitive pigment and the other two

were divided between the blue and yellow-green types. Such an arrangement might be an adaptation for high visual acuity, but to date no behavioral evidence concerning medium-wavelength visual acuity exists in the blowfly. Despite this interesting reasoning, more recent evidence (Trujillo-Ceno, 1965) indicates that eight sense cells are present in the ommatidia of flies.

In summary, it can be stated that while in the bee the spectral sensitivity curve of the receptor component of the mass response accurately reflects the sensitivity maxima of the individual receptor cells, this is not the case in the blowfly. The presence of the screening pigment is not the problem, for its effect on sensitivity can be overcome by attention to experimental design and correct analysis of results. The real problem is that the mass response cannot reflect sensitivity maxima of individual receptors which have (a) broadly overlapping sensitivity curves and (b) peaks varied in position and often separated by less than 50 nm or so. Both (a) and (b) are present in the blowfly.

(c.) mass response to heterochromatic flicker:

Despite the Munich group's cognizance of the effects of the screening pigment, they still could not accurately dismiss the 630 nm peak found by Autrum and Stumpf (1953) using the technique of heterochromatic flicker. In this technique the stimulus consists of a pair of wavelengths which are rapidly alternated; the relative intensities of the two wavelengths are adjusted until the transition

fails to evoke a "flicker potential" on the waveform. The experiments of Mazokhin-Porshnyakov (1960a, b) brought back into focus the problem of interpretation of the 630 nm peak based on the flicker potential. Similar to Autrum and Stumpf's technique, he flickered two lights against each other. One of these lights, the reference light, was itself composed of a mixture of two monochromatic lights which could be adjusted in intensity to abolish the flicker potential when alternated against the monochromatic test light. He moved the test light along the spectrum from 360 to 700 nm, and at each point adjusted the intensities of the violet and red lights in the reference combination until a match was obtained. The assumption was made that both reference wavelengths were in monochromatic regions of the spectrum, and that therefore he could plot the sensitivity curves of the dichromatic region between them by adjusting the intensity proportion of these two colors. He found that the region below 520 nm could be matched by the reference violet alone, and the region above 600 nm by the reference red alone. The lower "monochromatic" region of the spectrum had peaks at 365 nm and 490 nm, while the upper region had a peak at 630 nm; at high intensities the sensitivity curves of the two receptors crossed at 560 nm. However at low intensities the spectrum was monochromatic throughout. Similar data was obtained for Calliphora and Musca. Burkhardt (1962) found, however, that the violet end of the spectrum was in fact trichromatic. Nevertheless,

the problem of dismissing the 630 nm peak was not so easy, for other workers were not quite sure of the significance of the flicker potential. The matter was settled by Goldsmith (1965) who found in Musca that the on- and off-effects increase in prominence relative to the receptor component with (a) increasing intensity and (b) increasing wavelength; and that the spectral sensitivity curve of the off-effect follows exactly the sensitivity curve of Mazokhin-Porshnyakov's red receptor. These findings explain why the flicker potential yields a dichromatic curve only at high intensities, and provide evidence that this flicker potential is in fact unmatchable on- and off-effects.

(d.) the screening hypothesis---further evidence:

Utilizing white- and red-eyed Musca, Goldsmith (1965) provided further evidence of the validity of the screening hypothesis based on analysis of on- and off-effects, selective adaptation, energy-response curves, and responses to a microspot of light. Heretofore the screening hypothesis was based on absence of a red peak in action spectra from mass responses of white eyes and from single cell responses.

In selective adaptation experiments Goldsmith found that blue light accentuated (and red light depressed) the 620 nm peak in wild type flies. This classical evidence for a red receptor did not stand up in white eyes, however, where blue adaptation

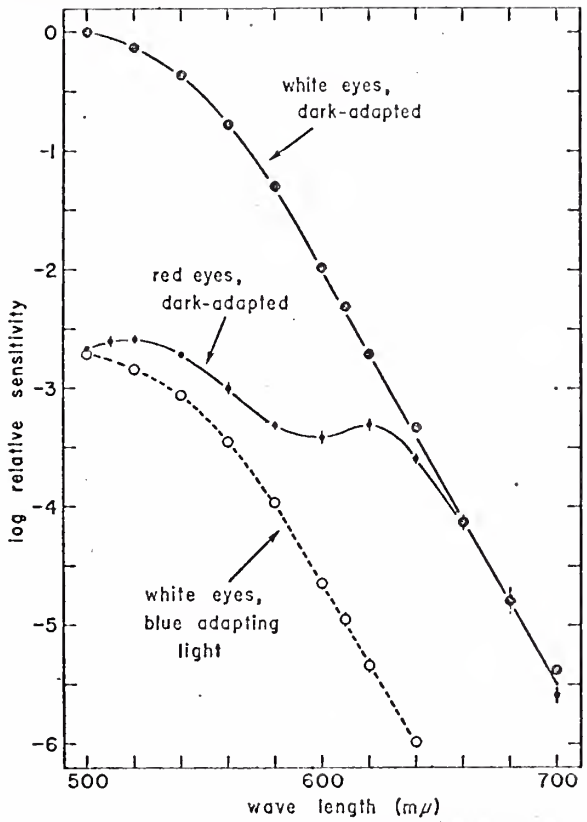

FIGURE 5. Spectral sensitivity curves of white- and red-eyed flies, both dark- and blue-adapted. Each curve represents average sensitivity curve of nine preparations. Note that the effect of the red screening pigment is essentially to (a) decrease the sensitivity of the receptors below about 600 nm, and (b) create a 620 nm sensitivity maximum for the ommatidial unit as a whole. (From Goldsmith, 1965)

failed to produce a long wavelength peak (Figure 5). Furthermore, the energy-response curves at varying wavelengths were found to be parallel in white-eyed flies, but in red-eyed flies to be steeper at 620 than at 500 nm. These findings can be explained by the ability of 620 nm light to pass readily through the screening pigment. Thus, in the adaptation experiment, the 620 nm peak, itself a product of the increased penetrance of the red test light and

consequent recruitment of peripheral receptors, is accentuated by blue adaptation of the green receptor system; in the white eye, however, no amount of blue adaptation can uncover a red receptor which doesn't exist. Likewise, the energy-response curve in red-eyed flies becomes steeper at long wavelengths as each incremental increase in intensity recruits additional peripheral receptors.

As mentioned in section (c), increase in intensity or wavelength of the stimulating light provokes an increase in the size of the off-effect relative to the receptor component. The increase in absolute size of the transients suggests recruitment of peripheral receptors; the increase in their relative size further suggests that the receptor component of axial cells is maximal, as defined by the sigmoid bend at the top of their energy-response curve, while peripheral cells are still being recruited. Definitive evidence that the off-response depends critically on the number, rather than the kind (Goldsmith, 1965) of cells stimulated was provided by comparing the size of the off-effect elicited by a 30μ spot of light with the size of the transient when the whole eye was stimulated, in red- and white-eyed flies. In white eyes the transients were larger when the whole eye was illuminated, at both 620 and 500 nm, while in red eyes this effect was present only at 620 nm. This ingenious experiment conclusively demonstrates (a) that red light is able to diffuse throughout the eye in red-eyed flies, and (b) that the size of the off-effect is

critically related to the number of receptors stimulated.

Whether these lessons from the bee and the blowfly apply to other insects will have to be determined in each individual case. It has been observed in these two animals that the visible spectrum extends from 300 to 650 nm, and that individual receptors may exhibit sensitivity maxima at 340-360, 430-470, 470-490 and 500-540 nm. The 620 nm peak in Calliphora may act functionally like the oil-droplet filter in reptiles to enable the animal to differentially perceive red light. The prediction, however, that visual acuity is sacrificed in the fly by this mechanism was substantiated by Schneider (1956) in behavioral experiments.

3. Other Insects

Information gleaned from investigation into the spectral sensitivity of other insects does not approach in completeness or accuracy that just described for Apis and Calliphora. The findings in the wasp, firefly, backswimmer, ground beetle, sphingid moth, dragonfly and butterfly will be reviewed, with particular reference to the presence of a red receptor in these animals.

On the basis of behavioral observation, Armbruster (1922) and Molitor (1939) concluded that the wasps Vespa vulgaris and germanica were able to distinguish red as a color. Schremmer (1939) noted that Vespa rufa could not distinguish between red and black pigmented papers.

Buck (1937) elicited a positive phototactic response in the

firefly Photinus pyralis to red light of wavelength 690 nm, and concluded that this animal was able to "visualize red stimuli".

Lüdtke (1954) measured the change in size of the mass response at several wavelengths equal in energy content in the backswimmer Notonecta glauca as the eye dark adapted after intense white adaptation. The dorsal and ventral halves of the eye were tested independently after two minutes and 30 minutes of dark adaptation. In the dorsal half the 575 nm peak receded and a 450 nm peak rose as dark adaptation proceeded; ventrally, the 535 nm peak increased in size and a 644 nm peak appeared. Of particular interest is the fact that the ventral part of the eye contains a crimson screening pigment (Rokohl, 1942); the time course of pigment migration and retinomotor movements (Lüdtke, 1953) was coincident with the appearance of the red sensitivity peak during dark adaptation. It thus appears likely that in this animal the red sensitivity peak is a consequence of differential absorption by the screening pigment, rather than a reflection of receptor sensitivity.

At high intensities, the spectral efficiency curves of the sphingid moth, Macroglossum stellatarum, and the ground beetle, Carabus auratus, exhibit peaks at 350 and 500 nm and a shoulder at 620 nm (Hasselmann, 1962). At low intensities, the 500 nm peak shifts to 440 nm, and the 620 nm shoulder decreases in prominence. This intensity dependence of the red shoulder again

suggests the possibility of a screening phenomenon.

Mazokhin-Porshnyakov (1959), using the technique of colorimetric analysis of flicker potentials in the dragonfly Libellula, found sensitivity maxima in the ventral half of the eye at 490 and 610 nm, and dorsally at 420 nm. As discussed in section (c) the flicker potential most likely represents unmatchable transients in the mass response. Such transients were shown in Musca to depend critically on the number of receptors stimulated; in the presence of a screening pigment with differential absorption, therefore, the flicker potential reflects the number of receptors reached by a particular wavelength more critically than the kind of receptors responding at that wavelength.

Finally, we come to a review of our current state of knowledge concerning the spectral sensitivity of diurnal butterflies, the animal on which the experiments to be reported upon in this paper were performed. Kühn and Ilse (1925) and Ilse (1928) studied the phototactic responses of four species to a field of variously colored paper flowers supported on glass stems. Only one species, Argynnis paphia, was attracted to red flowers at all, and in this instance a relatively small number of animals were attracted. Large numbers of Gonepteryx rhamni were attracted to purple flowers; since purple is a combination of red and violet, they may have responded to either of these colors. Small numbers of this species and Pieris brassicae were attracted to yellow flowers. In addition Pieris brassicae, Argynnis paphia and Vanessa io

responded strongly to blue flowers, and the last two of these species responded strongly to yellow flowers. The contribution that ultraviolet reflectance may have played in each of these instances is unknown.

More recently, Swihart (1963, 1964, 1965) has recorded equal energy spectra for various components of the mass response of the tropical diurnal butterfly Heliconius erato. This species had previously been studied behaviorally by Crane (1955) who noted that the animal responds preferentially in its feeding and courtship behavior to orange-red stimuli. Swihart found that the ERG's of Heliconius fall into two main types, which he called the "night" and the "day" responses. The former exhibits a large negative on-effect, a sustained negative receptor component, and a barely discernable negative off-effect; between the on-effect and the receptor component a transient decrease in negativity occurs which he calls the "dip". The day response, by contrast, exhibits a much larger dip, a receptor component with steeply increasing negativity, and a very large negative off-effect.

The spectral efficiency curve of the on-effect of both day and night responses has a major peak at 528 nm; the night response exhibits in addition a shoulder at 616 nm on a decreasing sensitivity curve.

The size of the dip (and thus indirectly, the size of the receptor component from trough to plateau) of day responses parallels the off-effect closely in size at varying wavelengths and

intensity. Equal energy spectra of these components were not recorded; rather, their size was expressed as a percentage of equal sized on-effects. Thus, the off-effect and receptor component relative to the on-effect increased in size as the test wavelength was increased from 420 to 528 nm to 616 nm. Thus, the (a) increasing size of the off-effect with increasing wavelength, and (b) the closeness with which the receptor component follows this wavelength-dependent off-effect, are both compatible with the screening hypothesis of etiology of long wavelength sensitivity. On the other hand, however, since the off-effect may vary with the kind of receptor stimulated, these findings are equally compatible with the presence of several classes of receptors.

In further experiments, Swihart (1964) anesthetized with carbon dioxide preparations which gave the night response and noted the conversion of the waveform to the characteristic day type response; i.e., carbon dioxide narcosis elicited the large off-effect and hyperpolarizing (dip) receptor component. As just noted, long wavelengths also accentuate the same components which differentiate day from night responses. Day (1941) observed that carbon dioxide narcosis in scotopic eyes caused movement of accessory pigments from their distal energy-requiring dark-adapted position to a proximal light-adapted screening position. Although photomechanical responses produced by light adaptation have not been studied in Heliconius erato, pigment migration in

pigment and retinular cells, nuclear migration in retinular cells and actual rhabdom migration have been observed in various other species in response to light adaptation (see Table I, p. 414, Goldsmith, 1964). Since (a) conversion of the night to the day type waveform in Heliconius erato occurs both as a circadian function and following carbon dioxide narcosis, and since (b) in other species carbon dioxide narcosis may produce retinomotor movements characteristic of the light-adapted eye, one might speculate that the long wavelength sensitivity of several components in the day response of Heliconius erato is somehow related to accentuation of a screening phenomenon by retinomotor movements. At any rate, given these observations, the burden of proof for the presence of a red receptor in this species is clearly on those who postulate its existence.

In this paper, experiments on the spectral sensitivity function of another butterfly, Colias eurythema, are reported, and preliminary observations on the possible existence of a red receptor are discussed.

II. MATERIALS AND METHODS

A. OPTICAL EQUIPMENT

1. The Test Light

The test source was an intense two mm arc generated by a Hanovia (D-901C-1) 150 watt xenon arc lamp operated at 7.5 amps from a DC power supply. The emission spectrum of this source extends throughout the visible spectrum and into the ultraviolet. A Bausch and Lomb grating monochromator (52mm square grating, 1200 lines per mm) with exit slits set at one mm provided a test beam with a half band width of 3.3 nm. This was used in conjunction with a columnator. The second order spectrum was cleared at 560 nm and longer wavelengths with a Corning CS3-72 filter. The intensity of the test beam was regulated with a pair of annular optical wedges capable of nearly four log units of attenuation. They consisted of films of inconel on quartz and were mounted so as to rotate in opposite directions thereby balancing each other. The degree of attenuation was (a) controlled by means of a finely machined drive shaft attached to these wedges, and (b) read off a continuous nine turn rotary dial calibrated to 0.01 revolution. When necessary these wedges were supplemented by single calibrated neutral density filters. Test flashes of duration 0.5 seconds were provided by means of a photographic shutter. The shutter was opened by a solenoid powered by eight 1.5 volt dry cells connected in series; this circuit was closed by

a relay box which could be triggered from a pulse generator at the appropriate time. The test flash was focused with a single quartz (to allow passage of ultraviolet) lens, such that an image of the diffraction grating just covered the extent of the cornea.

2. The Adapting Light

The adapting source was a six volt, fifteen watt, tungsten filament microscope lamp powered by a battery charger with which the intensity could be variably controlled. The wavelength content was regulated with filters (Zwiss RG-2; Corning CS0-51; 3-68; 5-56; 7-60). The adapting light was incident upon the eye at an angle approximately 30 degrees with the test light.

B. CALIBRATION OF THE QUANTAL OUTPUT OF THE OPTICAL SYSTEM

Calibration of the optical system consisted of determining (a) the log of the relative quantal output of the xenon arc lamp and monochromator unit at each wavelength setting, and (b) the log attenuation factor of the circular wedges over their entire range at each wavelength. Thus the output of the optical system at a given wavelength and wedge setting is equal to the sum of (a) and (b) and is called log relative quanta.

The energy of the xenon arc lamp and monochromator unit at each wavelength setting was determined by focusing the image of the diffraction grating on part of the quartz window of a twelve junction bismuth-silver Eppley thermopile. The thermopile output was amplified by a Keithley No. 149 milli-microvoltmeter and

recorded as linear deflections by a pen recorder. The deflection at each wavelength was expressed as a fraction of the largest deflection. The log of this ratio at each wavelength was added to the log of the factor for conversion of energy to quanta (since light is absorbed in quanta), to yield the total log quantal output of the light source at each wavelength. Because the short wavelength output of a xenon arc lamp decreases during the life of the unit, it was necessary to recalibrate the quantal output of the light source at two week intervals.

The log attenuation factor of the wedge system had been determined earlier at several wavelengths in our laboratory. A barrier layer photocell connected to the above described amplifier and recorder was used.

C. EXPERIMENTAL ANIMALS

"Clouded Yellow" butterflies, Colias eurythemae, were collected in a field in Bethany, Connecticut and maintained in cages in a temperature-controlled room with lighting automatically adjusted to simulate the natural photoperiod. They were fed each morning by mechanically lowering the proboscus onto a sponge soaked in a honey water (1:2) mixture. Seven male and female animals were used in the experiments.

D. THE RECORDING APPARATUS

The animals were decapitated and the proboscus, mouth parts and antennae removed. The head was placed on a tungsten

reference electrode which protruded through the upper surface of a cork platform such that the tip of the spike resided inside the head proximal to the optic ganglion. In one experiment, instead of a spike, the reference electrode consisted of a silver:silver chloride wire placed inside a glass pipette and connected to the contralateral cornea by means of a saline solution and a cotton wick. The receptor component of the retinal action potential recorded with this latter reference electrode was identical in shape to that recorded with the tungsten electrode, thus eliminating any doubt that the tungsten electrode might have been polarizing. An aluminum foil shield was placed over the head of all preparations to protect the contralateral eye from stimulation by stray light. A perforation was made in the shield large enough to admit light to the entire stimulated cornea, and the foil edges of the perforation were tamped down around the eye to prevent leakage of light behind the shield. The eye was positioned at the focus of the light source.

The active electrode consisted of a glass capillary pipette with a tip diameter of approximately 20μ, filled with an isotonic electrolyte solution of Ephrussi and Beadle (1936), and a silver: silver chloride wire was placed therein. The electrode tip was placed just under the cornea through a hole made with a fine insect pin. The active electrode was connected through (a) Bak unity gain single ended, and (b) Tektronix 3A3 direct coupled,

amplifiers to a Tektronix oscilloscope. The reference electrode was connected to ground. The preparation was electrically shielded by a Faraday cage.

A voltage calibrator in series with the reference electrode provided a 1mv x 100msec calibration pulse on the active trace prior to the response. A selenium photocell in the light path was connected to the second channel of the oscilloscope and provided a downward deflection on the trace under the active response, signalling the duration of the test flash. A bank of pulse generators was triggered by the opening of the camera shutter, and initiated after appropriate delays the CRO sweep, the closure of the test light shutter relay, and the calibration pulse. Responses displayed on the face of the CRO were photographed with a Grass 35mm camera on Kodak non perforated Linagraph Ortho (SP763 emulsion) film. The negatives were later projected onto a measuring surface by an enlarger.

The eyes were dark adapted for at least 20 minutes before testing to allow recovery from the light exposure sustained during preparation. In experiments requiring light adaptation, the eyes were exposed for at least 20 minutes prior to testing to provide assured adaptation. The test flashes were one half second. A minimum of 30 seconds separated test flashes to insure that the flash itself did not produce adaptation of the eye; a control experiment demonstrated that the responses to flashes at 30

second intervals did not decrease in magnitude over the series.

E. ANALYSIS OF DATA

The sensitivity of the eye is most completely expressed by comparing the relative slope and position of the energy-response curves at each wavelength. Since it is not practical to establish a complete family of E-R curves, one may simply measure a single curve, and assuming the others are parallel, determine the relative sensitivity at other wavelengths by means of a single test flash. If one suspects the curves may not be parallel, as in the present experiments, a spectral sensitivity curve may be constructed which is accurate at a given criterion response by extrapolating between several points on either side of the criterion. Criterion responses of one to three millivolts were used. Energy is expressed in terms of quanta, and the sensitivity of the eye as log relative sensitivity. The term relative signifies that absolute thresholds were not measured, but rather the relative number of quanta necessary to elicit a criterion sized response. Log relative sensitivity is thus equivalent to the inverse of log relative threshold.

III. RESULTS

A. THE RELATIONSHIP BETWEEN TRANSIENTS, RECEPTOR COMPONENTS, AND THEIR SPECTRAL SENSITIVITY CURVES

In only one animal (in which the reference electrode consisted of a wick on the opposite eye) were significant on- and off-effects observed. The on-effect may be seen at all wavelengths (Figure 6). The off-effect first appears (at high stimulus energies) at 460 nm; it does not become easily discernable, however, until 480 nm, and exceeds (in total height from the pre-stimulus baseline) the receptor component only at 520 nm and longer wavelengths.

The spectral sensitivity of these components is shown in Figure 7 for the eye in both the dark-adapted and long wavelength-adapted state. In the dark-adapted eye the receptor component exhibits a barely discernable shoulder at 360-380 nm, a peak at 440-450 nm, and a broad hump at long wavelengths. During orange light adaptation, the 440 nm maximum becomes prominent, and the broad hump splits into a small 560 and a larger 620 nm peak.

Perhaps the most significant feature of Figure 7 is the prominence of the long wavelength hump in the action spectrum of the receptor component. One might account for this hump by postulating the existence of (a) receptors at about 560 and 620 nm, (b) a single receptor with maximum sensitivity around 600 nm, or (c) a pigment screen, as in Musca, which allows

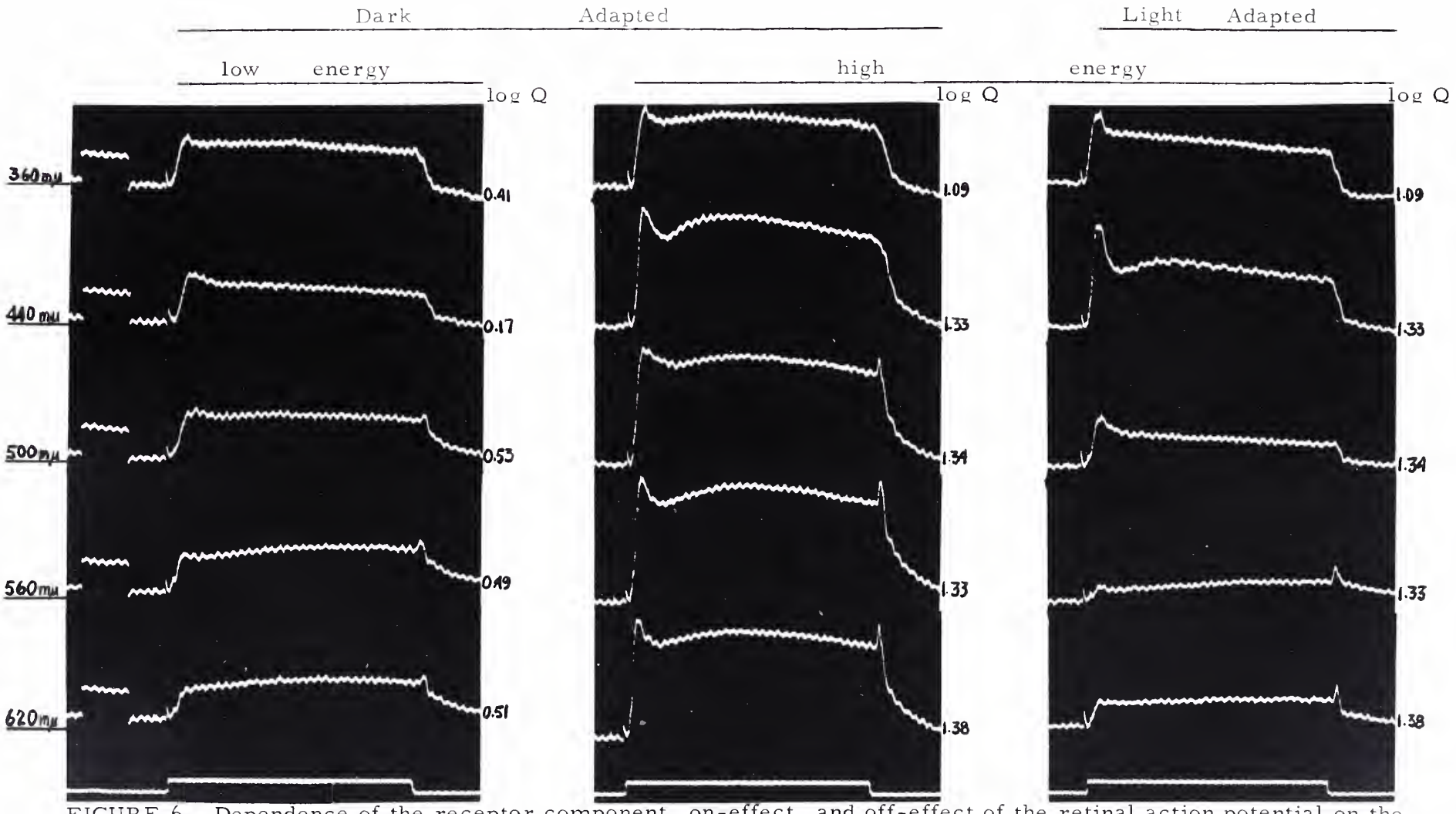

FIGURE 6. Dependence of the receptor component, on-effect, and off-effect of the retinal action potential on the wavelength and energy of the stimulus, and adaptation state of the eye. Responses recorded with subcorneal pipette, and wick reference electrode on the contralateral eye. Negativity of the active electrode is indicated by an upward deflection. Calibration pulse at left is 1mv x 100msec; photocell response under the traces is about 0.5 seconds. Relative stimulus energy, expressed as log quanta, is noted at the right of each response, and is approximately equal down each column. The responses in the second and third columns were elicited with equal stimulus energies; the average stimulus energy in these columns is 7.4 times the average in the first column. The first two columns are responses from the dark-adapted eye, the third from the eye adapted with long wavelength light. Wavelength of the test flash, noted at the left, is equal across each row.

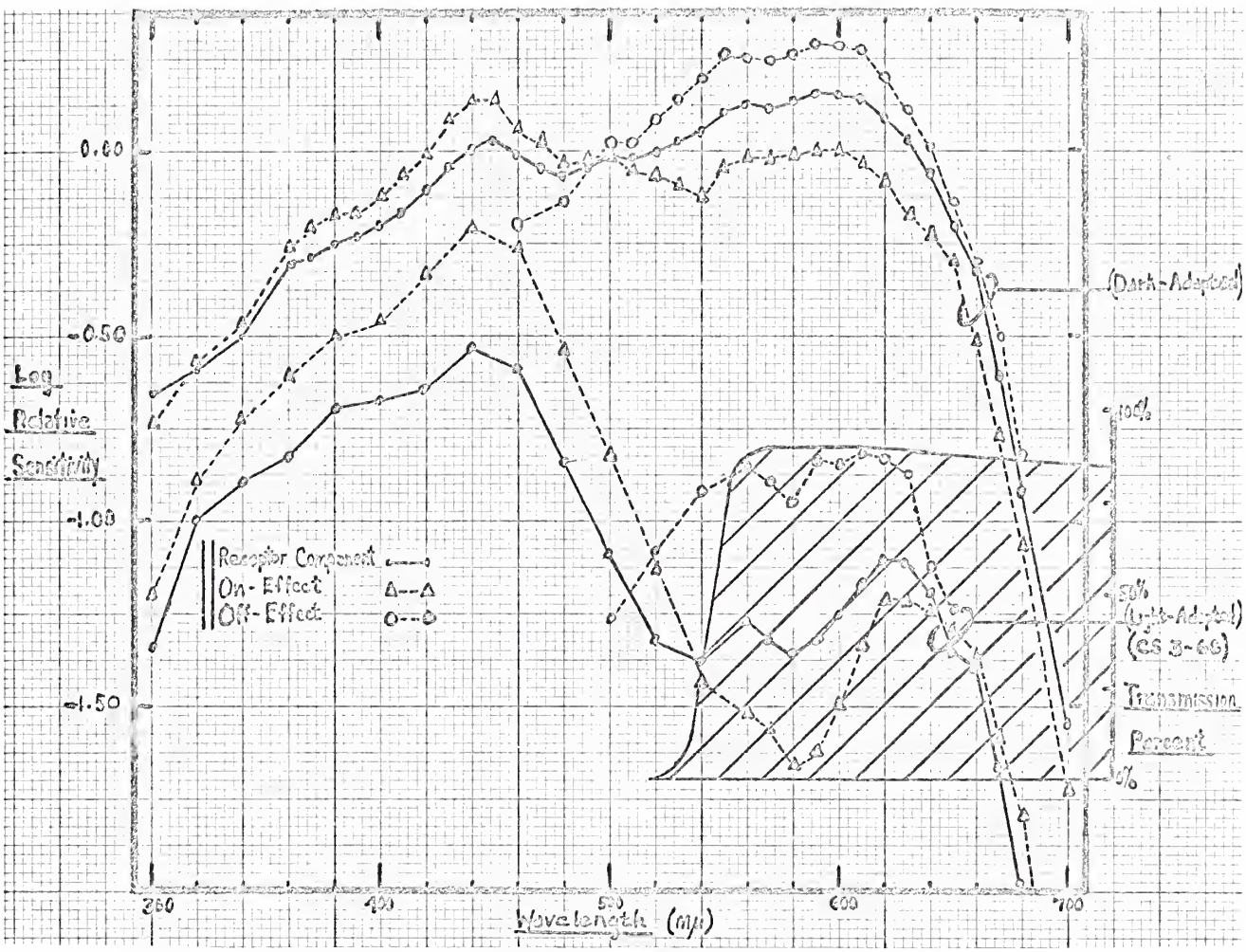

FIGURE 7. The spectral sensitivity curves of the receptor component, on-effect and off-effect of the retinal action potential of dark-adapted, and long wavelength adapted eyes. Criterion response equals 2.0 mv. Each point is based on the average of four responses. Cross hatched area represents transmission of adapting light.

preferential penetrance of long wavelength light. Simply the prominence itself of the long wavelength hump makes the third hypothesis unlikely, however. This is evident from an examination of Figure 5, in which the genesis of a red hump as a result of a screening phenomenon in Musca is described. In this fly the transmission spectrum of the screening pigment rises sharply at

600 nm (Strother, 1966); the red hump results because of a relative depression of sensitivity by 2.7 log units of the white-eyed action spectrum at wavelengths less than 600 nm. The red hump is thus a distortion of the downslope of the action spectrum of the green receptor in the fly, and its peak occurs at a lower relative sensitivity than that of the green maximum. A comparison of this situation with that portrayed in Figure 7 for Colias points up the dissimilarity of the red maxima. Thus for the red sensitivity of Colias to result from a screening effect, a substraction phenomenon of almost three log units would have to be present over the extent of the action spectrum in Figure 7 below 600 nm. When the Colias eye is cut open the pigment is dark brown or black in color; it is unlikely that such a pigment would possess a transmission spectrum, in view of the above considerations, capable of producing an action spectrum with the characteristics of long wavelength sensitivity seen in Figure 7. In summary then, the prominence and shape of the long wavelength maximum in Figure 7, as well as the evident lack of a bright red screening pigment, make the postulation of a long wavelength differential screening effect in Colias unlikely.

The splitting of the action spectrum (Figure 7) of the receptor component at long wavelengths after adaptation of the eye with orange light does not in itself allow one to decide whether one or two receptors exist in this region. The depression of the entire long wavelength end of the action spectrum suggests that the threshold

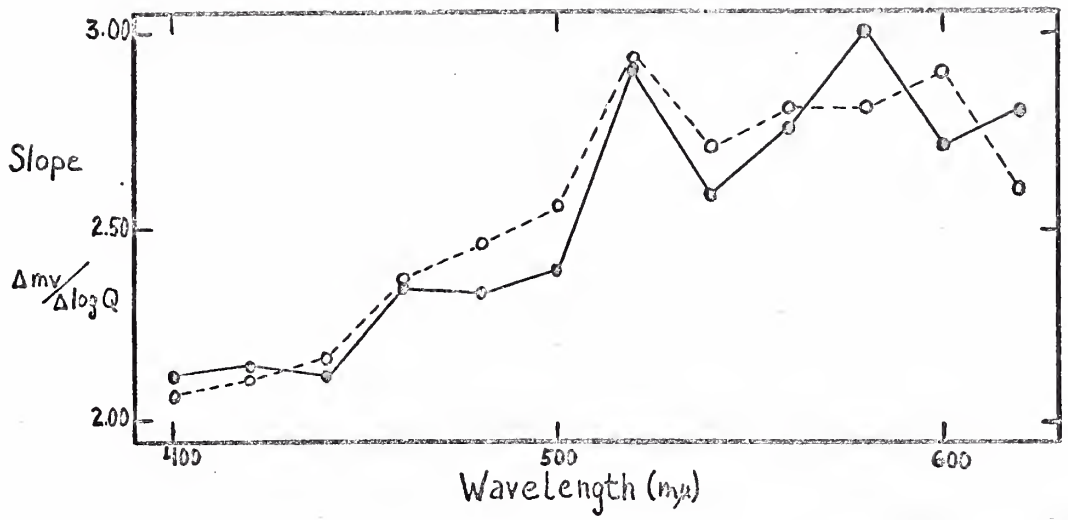

FIGURE 8. The slope of the energy-response curve of the receptor component at short vs. long wavelengths. Each point is based on four responses in the same eye. Solid circles and solid line: average slope of three lines between four response points at each wavelength. Open circles and dotted line: slope of energy-response curve (based on same four points) between 1.5 and 3.5 mv, at each wavelength.

of one or more receptors has increased. However no ready explanation exists for the distortion of the shape of sensitivity maxima by an adapting light which spans both maxima, since the shape of a sensitivity curve is determined by the absorption spectrum of a photopigment, which is unchanged by adaptation. In fact, Burkhardt and Hoffmann (1962) found spectral efficiency curves in Calliphora unaltered by adaptation.

In this experiment comparisons of complete E-R curves at short and long wavelengths were not made. Because four responses were elicited at each wavelength, however, a good approximation of the spectral variation of the E-R curves of receptor components has been constructed (Figure 8). The E-R curves

increase in steepness at long wavelengths. Goldsmith (1965) found a similar phenomenon in Musca, and demonstrated that it was a consequence of increased penetrance of red light through the screening pigment. Whereas blue light can stimulate only axial receptors, red light diffuses through the screening pigment and recruits, in addition, peripheral receptors. Thus each incremental increase in stimulus intensity elicits larger responses from axial receptors, and also provokes contributions from previously dormant peripheral units. An alternate explanation for nonparallel E-R curves is that different receptors are being stimulated. Red sensitivity may arise, then, as a result of either a red receptor or a differential screening effect; each possibility is an adequate explanation of the same phenomenon.

Figure 6 shows that the off-effect increases at long wavelengths, and Figure 7 that it exhibits a spectral sensitivity similar to the receptor component at long wavelengths in the dark-adapted eye. In the light-adapted eye, the difference between the sensitivity of the off-effect and the receptor component at long wavelengths is greater than in the dark-adapted eye. Table 1 provides evidence that the E-R curves of the off-effect and receptor component are parallel over the extent of the spectrum that they exhibit similar action spectra. At each wavelength, as stimulus energy is increased, the ratio of size of off-effect to size of receptor component remains fairly constant. The change in

Wavelength nm	Stimulus Energy Log Relative Quanta	Receptor mv	On-Effect mv	On-Effect On/Receptor	Off-Effect mv	Off-Effect Off-Receptor
440 nm	0.17	1.35	1.70	1.26	-	-
	0.58	2.15	2.55	1.19	-	-
	0.82	2.60	2.95	1.12	-	-
	1.33	3.90	4.15	1.06	-	-
460 nm	0.23	1.25	1.50	1.20	-	-
	0.65	2.20	2.55	1.16	-	-
	0.90	2.70	3.00	1.11	-	-
	1.42	4.15	4.50	1.08	3.45	0.83
500 nm	0.11	0.90	0.95	1.05	-	-
	0.53	1.65	1.75	1.06	1.65	1.00
	0.80	2.30	2.35	1.02	2.25	0.98
	1.34	3.90	4.10	1.05	3.77	0.97
540 nm	0.12	0.95	0.70	0.74	1.05	1.10
	0.55	1.70	1.30	0.77	1.85	1.09
	0.83	2.35	2.15	0.91	2.68	1.14
	1.37	4.35	4.45	1.02	4.65	1.07
580 nm	0.41	1.40	1.05	0.75	1.58	1.13
	0.69	2.00	1.70	0.85	2.15	1.07
	0.99	2.80	2.85	1.02	3.20	1.16
	1.25	3.90	4.10	1.05	4.30	1.10
620 nm	0.51	1.40	1.00	0.71	1.50	1.07
	0.83	2.15	1.90	0.88	2.40	1.11
	1.13	2.65	2.75	1.03	2.90	1.09
	1.38	3.75	4.10	1.09	4.00	1.06

TABLE 1. Changes in magnitude of receptor component, on-effect and off-effect at increasing stimulus energies at short and long wavelengths. Changes in ratio of transient:receptor component with changing stimulus energy at given wavelength reflect change of slope of E-R curve of transient relative to that of receptor component. Changes in size of this ratio at different wavelengths reflect shift in position of E-R curve of transient along energy axis relative to E-R curve of receptor component. Spectral change of slope of E-R curve of receptor component given in Figure 8.

magnitude of this ratio at varying wavelengths is a reflection of the shifting position along the energy axis of the E-R curve of the off-effect relative to the receptor component. This particular method of expression of position and shape of E-R curves of the transients, while lacking in quantitative and theoretical precision, is a fair approximation of the variation of these curves relative to the receptor component. Similar results were obtained at each wavelength by (a) averaging the $\Delta mv/\Delta \log Q$ of three lines connecting the four points, (b) determining the $\Delta mv/\Delta \log Q$ of the plotted E-R curve between 1.75 and 3.25 mv, and (c) comparing each of the three lines connecting the four points with the corresponding segment of the complete E-R curve at 500 nm, and averaging the net difference in displacement along the energy axis from the 500 nm E-R curve of the ends of each of the three lines.

What is the significance of these relationships between the off-effect and receptor component at long wavelengths in the dark- and light-adapted eye of Colias? Analysis of the off-effect in other animals has shown that it may be a function of several parameters. In Musca (Goldsmith, 1965) it is a function of the number of receptors responding; its E-R curve is parallel with that of the receptor component at 500 and 620 nm, and this curve is steeper at long wavelengths than short. To the extent that stimulation of larger numbers of receptors is responsible in Musca for a red peak in the action spectrum, one might expect that

incremental increases in stimulus energy would produce parallel E-R curves for the off-effect and receptor component. While the similar relationships in Musca and Colias of the E-R curves of the off-effect and receptor component suggest the possibility of a long wavelength screening phenomenon in Colias, they in no way preclude other explanations of red sensitivity. In the bee (Goldsmith, 1961b), where there is neither a screening effect nor a red peak in the action spectrum, the off-effect also increases at long wavelengths. Perhaps in the bee there is a lateral facilitative connection between the green receptors and those higher units which generate the off-effects. Such preferential neural connections could likewise exist with a red receptor. Since there is no evidence in Colias to indicate which of these mechanisms may underlie the enhancement of the off-effect at long wavelengths, it is not possible on the basis of the previous analysis of the relationship between the off-effect and receptor component to chose between the screening effect and red receptor theory of genesis of the red hump in the action spectrum of the receptor component of Colias.

The on-effect is present at all wavelengths (Figure 6), but at a criterion response of 2.0 mv demonstrates a spectral sensitivity quite different from the receptor component (Figure 7). Inspection of Table 1 reveals that its E-R curve (a) is parallel with that of the receptor component only at 500 nm, (b) is steeper at long, and shallower at short wavelengths, and (c) crosses the E-R

curve of the receptor component at high energy and long wavelengths. Its action spectrum thus depends on the criterion response chosen; if a criterion of four mv were used, its sensitivity at long wavelengths would be greater than that of the receptor component, rather than less as in Figure 7. The on-effect in Musca (Goldsmith, 1965) is difficult to compare with that in Colias because it is positive, rather than negative, in polarity. It has a shallow E-R curve at 500 nm and longer wavelengths; although its slope rises with increasing wavelength, it never exceeds that of the receptor component, attaining a maximum of only 2.8 mv at 500 nm. Although not measured at wavelengths less than 500 nm, its spectral sensitivity is similar to its counterpart in Colias at long wavelengths. The significance of the on-effect in Colias in terms of the screening versus the receptor hypotheses is unclear.

B. FURTHER ANALYSIS OF SPECTRAL SENSITIVITY FUNCTIONS

The retinal action potentials in the remainder of the experiments, recorded with a tungsten reference electrode inside the head, lack off-effects. Small energy dependent on-effects are present which increase with fatigue of the preparation and with strong light adaptation.

Figure 9 represents the spectral sensitivity curves of the receptor component of one such animal, during dark-adaptation, and adaptation with weak and strong red lights. The most striking feature of these curves are the prominent 350-370 nm sensitivity

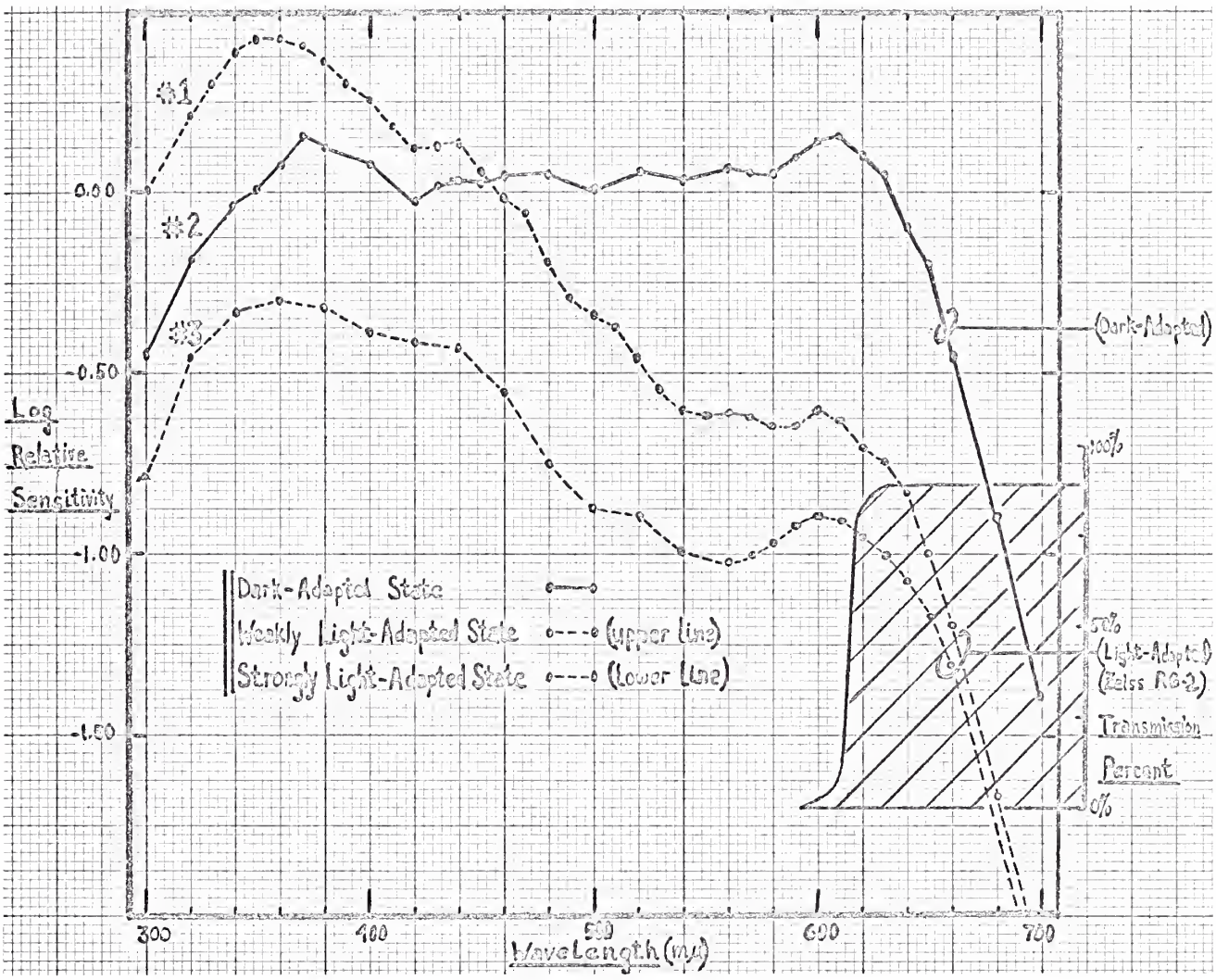

FIGURE 9. The spectral sensitivity curve of the receptor component of the retinal action potential. Criterion response equals 1.25 mv. Each point based on average of two responses. Cross hatched area represents transmission of adapting light. Numbers on curves refer to the order in which action spectra were recorded.

maxima; in addition, a shoulder is present at 440 nm. A steep long-wavelength sensitivity decline is produced by red adaptation. The ultraviolet sensitivity of curves #2 and #3 appears to have fatigued during the course of the experiment; this interpretation is based (a) on an increase of the on-effect from #1 to #2 (usually

dark adaptation produce a decrease of on-effect), and (b) on the paradoxically greater (0.42 log units) sensitivity drop between curves #1 and #3 at 360 nm (0.72 log unit drop) than at 600 nm (0.30 log unit drop) (red adaptation should produce a sensitivity gap greater at 600 than 360 nm). Thus the true sensitivity depression at long wavelengths during red adaptation is probably greater than represented by curve #3. The question of whether the 600-610 nm peak is a consequence of a red receptor or a screening phenomenon is again raised. Because the true sensitivity of the short wavelength end of the dark-adapted action spectrum is at least one half log unit greater than portrayed in curve #2, it is not possible here, as it was in Figure 7, to utilize the relative prominence of the red hump as an agrument in favor of a red receptor. In summary, Figure 9 shows peaks at 360 and 610 nm of similar sensitivity in the dark-adapted state. During red adaptation the long wavelength end of the curve is depressed; a 440 nm shoulder becomes evident, although the 360 nm peak remains relatively more sensitive.

The spectral sensitivity curve in Figure 10 gives evidence for a prominent peak at 380 nm, a broad hump around 500 nm and a smaller peak at 560 nm. Ultraviolet adaptation produces marked depression of the 360 nm peak and 500 nm hump, with shift of the short wavelength maximum to 390 nm. The 560 nm peak is relatively unaffected. These findings at the lower end of the spectrum

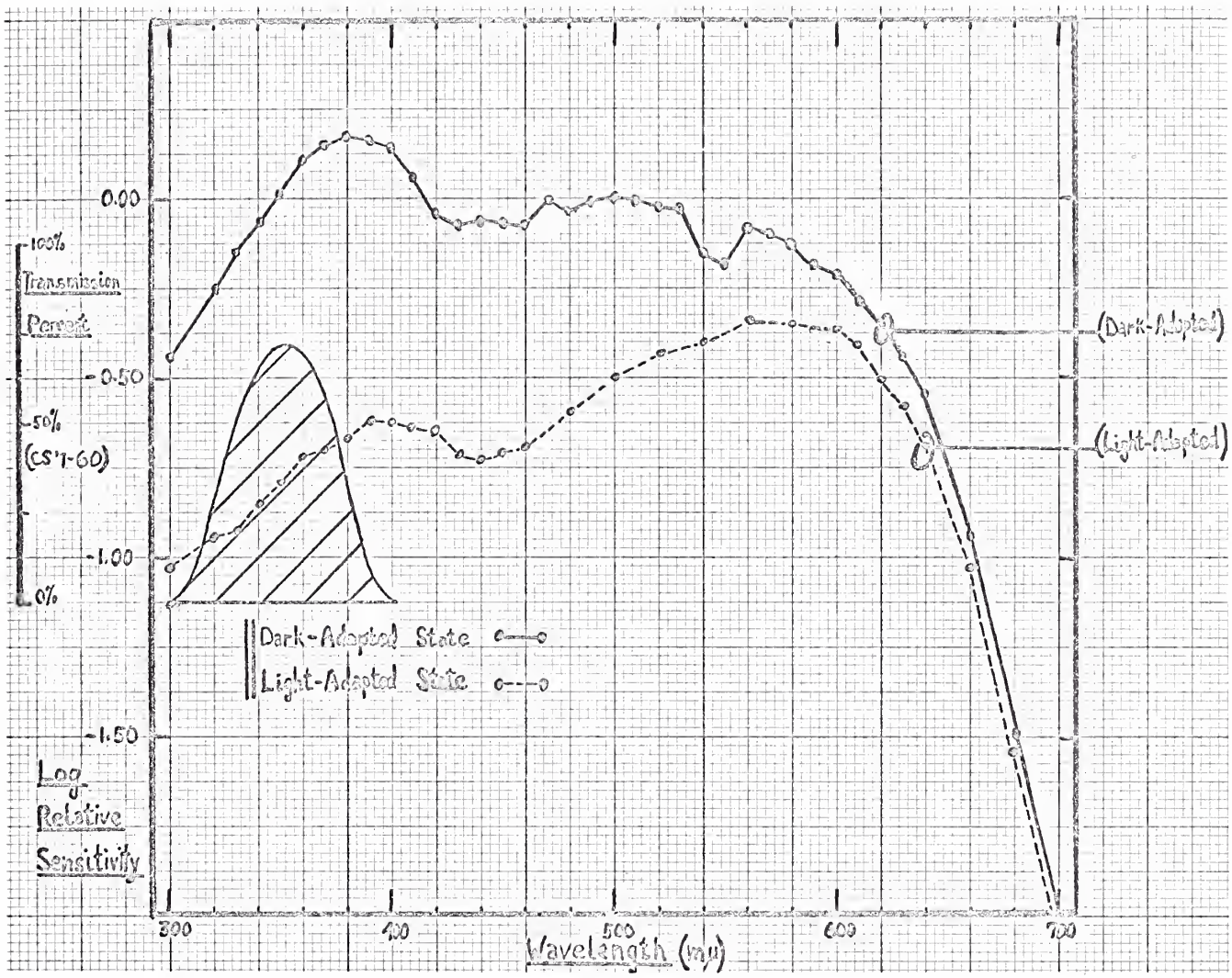

FIGURE 10. The spectral sensitivity curve of the receptor component of the retinal action potential. Criterion response equals 2.10 mv. Each point based on average of two responses. Cross hatched area represents transmission of adapting light.

are compatible with the existence of two kinds of receptors: one containing a photopigment with absorption maximum in the ultraviolet, and the other a pigment with maxima in both the blue-green and ultraviolet. A similar situation was found in the honey bee (Goldsmith, 1960). As the intensity of a green adapting light was

increased, the increment threshold of responses to green stimulation rose proportionately, while those to ultraviolet rose initially but quickly leveled off. These findings in the bee were confirmed at the cellular level by Autrum and von Zwehl (1962) and Autrum (1963) who found both an ultraviolet receptor, and a green receptor with secondary sensitivity in the ultraviolet (Figure 3). Similarily, in Calliphora (Burkhardt, 1962), 440, 470 and 520 nm receptors were found to have significant ultraviolet sensitivity (Figure 4). In order to confirm that this situation exists in Colias, as suggested by Figure 10, it would be necessary to demonstrate that blue-green adaptation increases the relative prominence of the ultraviolet peak. Such an effect was hinted at in two other experiments designed with this objective in mind, but due to imprecise filter choice, the results were not conclusive. The shape of the action spectrum in Figure 10 is different than those in Figures 7 and 8. In fact the action spectra were found to vary from one preparation to the next in Colias, although no maxima other than those already described were elicited. A similar situation has been found in the drone bee (Goldsmith, 1960). These findings suggest that the proportion of receptor types varies throughout the eye in Colias, and that the difference in action spectra may depend on the proportion of receptor types which happen to be aimed at the stimulus beam.

IV. DISCUSSION

Further experiments will contribute substantially to our understanding of color vision in Colias. Records from the optic ganglion will help us understand the significance of the various components of the retinal action potential at a more integrated level. More data on transients is necessary. More dark-adapted action spectra are needed, as well as action spectra during adaptation with fairly narrow band interference filters. Hopefully it will be possible to measure the absorption spectrum in vivo of the secondary pigment screen. Such data will go a long way toward assaying the validity of our intellectual constructs concerning the selective light transmission of these pigments. Experiments in which the lateral diffusion of long wavelength light is restricted by means of a microspot or fiber-optics conduction will provide a more accurate idea of the spectral sensitivity of retinular clusters, as opposed to ommatidia. In the final analysis, intracellular recording using axial illumination will provide definitive action spectra of individual receptors.

The retina of Colias, as with all other insects tested by electrophysiological means, is sensitive to light from 300 to 650 nm. Insects tested with the technique of intracellular recording to date possess a trichromatic system of color vision. The general rule then that insects, like vertebrates, possess a trichromatic system of color vision, and that this system is shifted approximately 100 nm

towards shorter wavelengths, has not been conclusively violated. The presence of a differential screening mechanism in an animal which lacks a red receptor might cause one to question the functional significance of such a system for the animal. The red peak in the action spectrum of flies suggests that this animal can indeed differentially perceive red light. Observations of feeding and mating behavior lend support to this suggestion. Schneider (1956) found by behavioral means that visual acuity in the fly is lower in the red than in other regions of the spectrum. Evidence does exist, then, to support the theoretical prediction that attainment of differential red sensitivity by means of a screening phenomenon necessarily entails the sacrifice of visual acuity in this region.

Swihart (1963, 1964, 1965) has set forth indirect evidence (section I, D, 3) proposing the existence of a red receptor system in the butterfly Heliconius. Neither in Heliconius nor in any other insect, however, in which a red receptor system has been proposed, has evidence foran ultraviolet receptor system been set forth to date. This paper suggests that Colias indeed may possess a red receptor, and furthermore, that this butterfly does possess differential ultraviolet sensitivity.

V. CONCLUSIONS

1. Retinal action potentials may be elicited in the eye of Colias over the spectral range 300 to 700 nm.

2. Spectral sensitivity maxima are variously present at 360-380, 440, 500, 560 and 600-620 nm.

3. The presence of a red receptor is suggested by analysis of the shape and relative sensitivity of the long wavelength maximum in the dark-adapted action spectrum, as well as by observation of the color of the pigment in the freshly cut eye.

4. Ultraviolet adaptation produces marked depression of the ultraviolet peak and shift of its maximum to longer wavelengths, as well as depression of the 500 nm hump. Green adaptation on the other hand, appears to depress selectively the latter hump. These observations suggest that the Colias eye possesses both ultraviolet and green receptors, and that the latter has secondary absorption in the ultraviolet.

5. An off-effect is present at long wavelengths; its action spectrum parallels closely that of the receptor component. The slopes of the energy-response curves of the off-effect and receptor component increase in a parallel fashion at long wavelengths.

6. An on-effect is present at all wavelengths. Its size is a function of adaptation state as well as stimulus energy and wavelength. Its energy-response curve in the dark-adapted state is parallel to the curve of the receptor component only at 500 nm; at short wavelengths its slope is shallower and at long wavelengths steeper.

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